**Proreceptor Dimerization Is Required for Insulin Receptor Post-translational Processing**

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The insulin receptor is a transmembrane protein dimer composed of two αβ monomers held together by inter-α-chain disulfide bonds. In a previous report we described a monomeric insulin receptor obtained by replacing Cys-524, -682, -683, and -685 with serine (Wu, J. J., and Guidotti, G. (2002) J. Biol. Chem. 277, 27809–27817). The membrane-bound monomeric insulin receptors could be cross-linked to dimers in the presence of insulin, indicating that although covalent interactions had been abolished, noncovalent dimerization could still occur in the membrane. To eliminate noncovalent dimerization, we replaced all or some of Cys-524, -682, -683, and -685 with arginine or aspartic acid with the expectation that the electrostatic repulsion at these contact sites would prevent noncovalent dimerization. The results indicate that mutant insulin receptors that are able to form covalent dimers are expressed at the wild type level; mutants that can form noncovalent dimers are expressed at half the level of the wild type receptor, whereas insulin receptor mutants that cannot dimerize are expressed at less than 10% of the wild type level. To elucidate the mechanism of the decrease in expression of the mutant insulin receptors, we examined their subcellular localization and biosynthesis. The results suggest that the extent of expression of these mutant receptors is related to their ability to form covalent or noncovalent dimers at the proreceptor stage.

The insulin receptor (IR) is a glycosylated membrane protein composed of two αβ monomers (1). The α and β subunits are derived from a single proreceptor precursor. During insulin receptor maturation two proreceptors undergo a series of post-translational processing steps, including disulfide bond formation, proteolytic cleavage, and glycosylation, to form a mature dimeric receptor on the cell surface (2–5). Mutations that affect IR processing have been extensively studied (for review, see Ref. 6).

The insulin receptor subunits are linked by disulfide bonds (7). Although there are 37 cysteine residues in the α subunit and 10 cysteine residues in the β subunit, only one disulfide bond links the α (Cys-647) and β (Cys-872) subunits in the monomer. This link is a class II disulfide bond, which is disrupted only under severe conditions (detergents like SDS in addition to reducing agents) (8, 9). Cys-524 and at least one of the cysteine residues of the triplet at positions 682, 683, 685 are involved in the dimerization of two αβ monomers. The disulfide bonds between two αβ monomers are classified as class I disulfide bonds that are disrupted by mild conditions (reducing agents only) (9–15). We have recently constructed a monomeric insulin receptor by replacing Cys-524, Cys-682, Cys-683, and Cys-685 residues with serine; the IR monomer was autophosphorylated in response to insulin in intact cells and membranes (16). However, membrane-bound monomeric insulin receptors could be cross-linked to dimers in the presence of insulin, suggesting that the monomeric insulin receptors, IR(C524S,C682S,C683S,C685S) need to dimerize noncovalently to undergo autophosphorylation. To construct an insulin receptor that would not dimerize noncovalently we replaced the four cysteine residues Cys-524, Cys-682, Cys-683, Cys-685 with arginine (Arg), aspartic acid (Asp) or tryptophan (Trp). We expected that the side chain electrostatic repulsion (from Arg and Asp) and the steric hindrance (from Trp) might prevent noncovalent dimerization of the monomers and allow us to study the properties of monomeric IR. Surprisingly, the IR(C524R,C682R,C683R,C685R) and IR(C524D,C682D,C683D,C685D) mutants were expressed at very low levels, 6% of IRWT, and there was almost no proreceptor in the endoplasmic reticulum-Golgi complex. On the other hand replacement of Cys-524 with Trp was sufficient by itself to prevent processing of the mutant IRs, which were retained in the endoplasmic reticulum-Golgi complex as proreceptors. The conclusion is that proreceptor dimerization is required for IR processing and movement to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), LipofectAMINE, fetal bovine serum, and phosphate-buffered saline (PBS) were purchased from Invitrogen. The sources of antibodies were Invitrogen (Carlabar, CA) for anti-V5 antibody, Oncogene Research Products (Cambridge, MA) for insulin receptor antibody Ab-1, StressGen Biotechnologies Corp. (Victoria, Canada) for antibody SPA-860 against calnexin, and this laboratory for antibody 620 against the IR, and 125I[Insulin and [35S]Met/Cys (Express-35S) were from Sigma; Protran nitrocellulose membrane was from Schleicher & Schuell, Inc.; Supersignal West Pico chemiluminescent substrate was from Pierce; gradient polyacrylamide gel (4–15%) was from Bio-Rad. DNA sequencing was performed with an ABI Prism 310 Genetic Analyzer made by Applied Biosystems (Foster City, CA).

Construction of Plasmids—Plasmid pJW17, which contained the full-length human IR cDNA in the LITMUS28 vector (16), was used in these experiments. Site-specific mutations of Cys-524 were introduced by mutagenesis primers GGGTCATTGGTCTACACCCGTTACACTTTGGG-AACcGgGACATCCTGC (C524R mutation), GGGTCATTGGTCTACACCCGTTACACTTTGGG-ACcGgGACATCCTGC (C524D mutation), and GGGTCATTGGTCTACACCCGTTACACTTTGGGC-ACcGgGACATCCTGC (C524W mutation), and GGGTCATTGGTCTACACCCGTTACACTTTGGG-ACcGgGACATCCTGC (C524D mutation). To introduce mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation). To introduced mutations at Cys-682 to Cys-685, the following primers were used: ATGAGGATTCGGCCGCGAAC-CTGC (C524D mutation).
GTGCTCTGTGCCTAA (C682R mutation), ATGGAGGATCCGGCCGG-
CGAACGCTGCTGTTCAA (C682R, C683R, C685R mutation), ATG-
AGGATCCGGCCGGCAGAAGAGCTTCCGTCACA (C682D, C683D,
C685D mutation). The nucleotides involved in mutagenesis are indi-
cated in lowercase. The entire PCR fragments were sequenced to verify
that no other base changes occurred. The mutated sequences were cut
and pasted into the corresponding regions in mammalian expression
plasmids containing wild type or mutant insulin receptors (16). All IR
constructs contained a V5 epitope tag at the C terminus of the 
β-subunit.

Cell Culture and Transfection—COS7 cells were maintained with 5%
CO₂ at 37°C in DMEM supplemented with 10% fetal bovine serum. To
transiently express the insulin receptors, subconfluent cells were trans-
formed with plasmid DNA and LipofectAMINE according to the manufa-
cturer’s instructions.

Insulin Binding Assays—COS7 cells were transfected with plasmids
harboring different forms of insulin receptors. Forty-eight hours after
transfection, the cells were washed twice with ice-cold PBS.

[125I]Insulin (50 pmol) was incubated with the cells for 12 h at 4°C in a
binding buffer containing 100 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM
KCl, 1.2 mM MgSO₄, 10 mM glucose, and 1% bovine serum albumin.
After incubation, the cells were washed 3 times with ice-cold binding
buffer, solubilized in 0.1% SDS, and counted in a γ counter. Nonspecific
binding was determined by including 10 μM unlabeled insulin in the
incubation. Dissociation constants were determined by Scatchard plot
analysis of [125I]Insulin binding to crude membranes, as previously
described (16).

Western Blot Analysis of Insulin Receptors—Western blot analysis
was performed as described previously (16). Briefly, COS7 cells express-
ing insulin receptors were solubilized with Laemmli buffer under non-
reducing or reducing conditions (with 50 mM N-ethylmaleimide or 5%
β-mercaptoethanol). The proteins were separated by SDS-PAGE, trans-
ferred to a nitrocellulose membrane, and immunoblotted with anti-V5
antibody. Densitometric analysis of the signals was performed using
NIH image software.

Metabolic Labeling—COS7 cells were transfected with plasmids con-
taining mutant IRs. Forty-eight hours after the cells were washed twice
with Met/Cys -DMEM followed by incubation with Met/Cys -DMEM
plus 250 μCi/ml [35S]Met/Cys (Express-35S35S) for 30 min. The cells were
then washed and chased in complete DMEM medium. At various time
points as specified in Fig. 5 the cells were washed and solubilized with
lysis buffer (0.5% Nonidet P-40 in 50 mM Tris-HCl, pH 7.5, 200 mM
NaCl, 2.5 mM β-mercaptoethanol, 1 μM leupeptin, 1 μM chymostatin, and
1 μM pepstatin) and immunoprecipitated with anti-IR antibody
(Ab-1) and protein G-agarose beads. The precipitated IRs were released
from the beads with Laemmli buffer in non-reducing condition, sepa-
rated on a 4–15% gradient polyacrylamide gel, and analyzed by a Bio-
Rad Molecular Imager FX System. The 35S-labeled insulin recep-
tors used for two-dimensional gel electrophoresis were generated in a
similar manner, except that the 35S-labeling time was longer with no
chase.

Two-dimensional Gel Electrophoresis—[35S]Met/Cys (Express-35S35S)-
labelled insulin receptors were released from protein G-agarose beads
with Laemmli buffer under non-reducing condition and separated on a
3–10% polyacrylamide gel. A lane of gel containing cell proteins was cut
and treated in Laemmli buffer with 5% β-mercaptoethanol at 55°C for
20 min. The gel slice was then put on a 7% polyacrylamide gel, and analyzed by
a Bio-Rad Molecular Imager FX System. The 35S-labeled insulin recep-
tors used for two-dimensional gel electrophoresis were generated in a
similar manner, except that the 35S-labeling time was longer with no
chase.

Preparation of Crude Membranes—Forty-eight hours after transfection,
five 100-mm plates of transfected COS-7 cells were washed twice
with 10 ml of PBS containing 137 mM NaCl, 2.7 mM KCl, 8.0 mM
Na₂HPO₄, and 1.5 mM KH₂PO₄. The cells were scraped off the plates
with a rubber policeman after a 5-min incubation in PBS supplemented
with 5 mM Na₂EDTA, pelleted by centrifugation at 10,000 × g at 55°C for 3 min,
and resuspended in 2 ml of ice-cold HEPES buffer (10 mM, pH 7.4)
containing 50 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 2.5
μM β-mercaptoethanol, 1 μM leupeptin, 1 μM chymostatin, and 1 μM
pepsatin. The cells were homogenized in 25 strokes in a Dounce
homogenizer with a tight pestle followed by adding 264 μl of 65%
sucrose (w/w) in 10 ml HEPES, pH 7.4, 4 μl of 0.5 M MgCl₂, 13.2 μl of
2.5 μM β-mercaptoethanol. The homogenate was then subjected to two 10-min
spins at 1000 × g at 4°C to remove nuclei, mitochondria, and unbroken
cells. Crude membranes were pelleted from the resulting supernatant
by centrifugation at 150,000 × g in a Beckman Ti70.1 rotor for 30 min.

They were then washed quickly in 2 ml of HEPES buffer and resus-
pended in the same buffer. Protein concentration was determined using
Bio-Rad (Bradford) protein assay kits. The membranes were quickly
frozen in liquid nitrogen and stored at −70°C for further analysis (17).

Crude Membrane Fractionation—Crude membrane protein (100 μg)
was resuspended in 0.7 ml of 10 mM HEPES, pH 7.4, homogenized with
15 strokes in a Thomas Teflon pestle homogenizer, combined with 2.3
ml of 65% (w/w) sucrose in 10 mM Hepes, pH 7.4, and placed at the
bottom of an SW40Ti centrifuge tube. The sample was subsequently
overlaid with 1 ml each of 45, 40, 35, 30, 25, 20 15, and 10% sucrose
solutions (w/w in 10 mM Hepes, pH 7.4). After centrifugation at
85,000 × g for 18 h, the fractions (0.75 ml/fraction) were collected from
the top of the tube. The sucrose concentration in each fraction was
determined with an Abbe refractometer. The fractions were diluted in 6
ml of ice-cold PBS and centrifuged at 150,000 × g for 30 min in a Beckman
Ti70.1 rotor to pellet the membranes. The membranes were solubilized with
Laemmli sample buffer containing 5% β-mercaptoethanol and 100 mM
dithiothreitol, subjected to SDS-PAGE, and immunoblotted with
antibodies. The plasma membrane and endoplasmic reticulum
fractions were determined by immunoblotting with an anti-
α1 (Na,K-ATPase) antibody and an anti-calnexin antibody, respectively
(17).

FIG. 1. Immunoblots of wild type and mutant insulin recep-
tors. COS7 cells expressing wild type or mutant insulin receptors were
solubilized with Laemmli buffer under nonreducing conditions. Solubi-
lized proteins were separated on a 4–15% gradient polyacrylamide
gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-V5
antibody. Lane 1, IRWT; lane 2, IRC524D; lane 3, IRC524D,IRC524R;
lane 4, IRC524D,IRC524R,IRC524D; lane 5, IRC524D,IRC524R,IRC524D,
IRC524D,IRC524D; lane 6, IRC524D,IRC524R,IRC524D,IRC524D,IRC524R,
IRC524D; lane 7, IRC524D,IRC524R,IRC524D,IRC524D,IRC524R,IRC524D; lane 8,
IRC524D,IRC524R,IRC524D,IRC524D,IRC524R,IRC524D,IRC524D; lane 9, COST cells without transfection. The lower
panel was exposed 10× longer than the upper panel to show the presence of
some monomer and proreceptor in lanes 7 and 8.
COS7 cells were transfected with plasmids containing the cDNAs from the wild type and mutant insulin receptors indicated in the table. 

**Table I**

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<th>Dissociation constants and insulin binding sites</th>
<th>Binding sites/cell</th>
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<tr>
<td>1. IRWT</td>
<td>3.05 × 10⁻⁹ (6.59 × 10⁻¹¹)</td>
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<tr>
<td>2. IRIC524R</td>
<td>3.54 × 10⁻⁹ (9.19 × 10⁻¹¹)</td>
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<tr>
<td>3. IRC524R,C682S,C683S,C685S</td>
<td>2.82 × 10⁻⁹ (7.76 × 10⁻¹¹)</td>
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<tr>
<td>4. IRC524R,C682D,C683D,C685D</td>
<td>3.52 × 10⁻⁹ (9.13 × 10⁻¹¹)</td>
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<tr>
<td>5. IRC524R,C682R,C683R,C685R</td>
<td>3.46 × 10⁻⁹ (9.10 × 10⁻¹¹)</td>
</tr>
<tr>
<td>6. IRC524R,D</td>
<td>3.73 × 10⁻⁹ (9.39 × 10⁻¹⁰)</td>
</tr>
<tr>
<td>7. IRC524R,D,C682D,C683D,C685D</td>
<td>3.30 × 10⁻⁹ (9.56 × 10⁻¹¹)</td>
</tr>
<tr>
<td>8. IRC524R,C682S,C683S,C685S</td>
<td>3.73 × 10⁻⁹ (9.39 × 10⁻¹⁰)</td>
</tr>
<tr>
<td>9. IRC524R,C682R,C683R,C685R</td>
<td>3.30 × 10⁻⁹ (9.56 × 10⁻¹¹)</td>
</tr>
<tr>
<td>10. IRC524R,C682S,C683R,C685R</td>
<td>3.30 × 10⁻⁹ (9.56 × 10⁻¹¹)</td>
</tr>
<tr>
<td>11. IRC524R,C682D,C683D,C685D</td>
<td>3.28 × 10⁻⁹ (9.53 × 10⁻¹¹)</td>
</tr>
<tr>
<td>12. IRC524R,C682D,C683D,C685D</td>
<td>3.28 × 10⁻⁹ (9.53 × 10⁻¹¹)</td>
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Insulin binding as percent of the amount bound to cells expressing IRWT

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<th>Binding sites/cell</th>
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<tr>
<td>13. IRIC524R,C682S,C683S,C685S</td>
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<tr>
<td>14. IRC524R,C682D,C683D,C685D</td>
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<tr>
<td>15. IRC524R,C682R,C683R,C685R</td>
</tr>
<tr>
<td>16. IRC524R,C682S,C683R,C685R</td>
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<td>17. Mock transfection control</td>
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The values in brackets are the insulin binding sites per cell assuming that the dissociation constant for insulin of the mutants is similar to that for IRWT.

**Fig. 2.** C524W mutation significantly affects IR maturation. COS7 cells expressing IRWT or IRC524W were solubilized with Laemmli buffer in nonreducing conditions. The proteins were separated on a 3–10% gradient polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-V5 antibody. Panels A and B are from the same experiment, and panel B was exposed 10 times longer than A.

**Fig. 3.** Immunoblots of monomeric insulin receptors. COS7 cells expressing monomeric mutant insulin receptors were solubilized with Laemmli buffer in nonreducing conditions. The proteins were separated on a 4–15% gradient polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-V5 antibody. Lane 1, IRC524R,C682S,C683S,C685S; lane 2, IRC524R,C682R,C683R,C685R; lane 3, IRC524R,C682S,C683R,C685R; lane 4, IRC524R,C682S,C683S,C685R; lane 5, IRC524R,C682D,C683D,C685D; lane 6, IRC524R,C682D,C683S,C685D; lane 7, COS7 cells without transfection.

Immunochemistry Staining—COS7 cells transfected with plasmids containing wild type or mutant IR cDNA were grown on coverslips. 48 h after transfection, the cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with ice-cold methanol for 2 min. After washing with 0.1% Triton X-100, PBS 3 times, the coverslips were blocked with 10% goat serum, PBS for 30 min and stained with either anti-V5 antibody or anti-calnexin antibody followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG or Cy3-conjugated anti-mouse IgG secondary antibodies for 30 min. DNA was stained with 4,6-diamidino-2-phenylindole to localize the nucleus. The secondary antibodies were removed from the coverslips by washing with PBS 3 times, and the coverslips were exposed to fluorescein isothiocyanate-conjugated anti-rabbit IgG or Cy3-conjugated anti-mouse IgG secondary antibodies for 30 min. The samples were then washed with PBS 3 times and observed under a Zeiss LSM510 confocal microscope.

**RESULTS**

Expression of the Mutant IRRs in COS7 Cells—To make IR monomers that do not dimerize noncovalently, we placed...
charged (Arg or Asp) or bulky (Trp) residues at the positions of cysteine residues 524 and 682, 683, 685 to prevent interactions between the α chains.

Because amino acid substitutions of a membrane protein can affect the structure of the protein leading to activation of the endoplasmic reticulum quality control system (18), we first determined whether substitutions at either position 524 or 682, 683, 685 alone were tolerated by the IR. COS7 cells were transfected with plasmids expressing wild type and mutant IRs. Forty-eight hours after transfection, insulin binding to intact cells and to crude membranes was carried out to determine the dissociation constants for insulin and the number of insulin binding sites per cell. The results are shown in Table I.

Fig. 1 shows the results of experiments to determine whether the IRs are present as dimers or monomers. It is evident that replacement of Cys by Ser, Arg, or Asp at either position 524 or at 682, 683, 685 alone was tolerated by the IR. COS7 cells were transfected with plasmids expressing wild type and mutant IRs. Forty-eight hours after transfection, insulin binding to intact cells and to crude membranes was carried out to determine the dissociation constants for insulin and the number of insulin binding sites per cell. The results are shown in Table I (lines 2–7) and that in all these cases IR dimers were present (Fig. 1, lanes 2–5). The presence of insulin receptor dimers of the mutants IR\text{C524S}, IR\text{C682S}, IR\text{C683S}, IR\text{C685S} were reported in Lu and Guidotti (15) (see Fig. 1B, lane 4) and in Cheatham and Kahn (8) (see Fig. 3, lane 6), respectively. Clearly, the substitution of Ser, Arg, and Asp for Cys did not affect processing, appearance at the cell surface, and insulin binding of these mutant receptors. We conclude that as long as cysteines are present either at position 524 or at 682, 683, 685 interchain disulfide bonds can form between the α chains even if there is charge repulsion at the other contact site. We also conclude that the presence of interchain disulfide bonds allows proper processing of the IR. In contrast, Trp at position 524 was highly deleterious because there was little insulin binding at the cell surface (Table I, line 15), the IR proreceptor was not significantly processed (Fig. 2A), and there was minimal IR dimer (Fig. 2B). Only when the immunoblot was exposed for a long time at least three distinctive bands were observed in the position of the mature dimeric insulin receptor (Fig. 2B). Clearly, processing was severely affected in this case, supporting the view that the lack of insulin binding at the cell surface was caused by a lack of surface receptors rather than a change in the dissociation constant for insulin.

The next set of IR mutants was made by combining the substitution of either Asp or Arg at one position with Ser at the other position. The idea here is that whereas there might be electrostatic repulsion at one α–α interface, at the other interface noncovalent interactions can take place. The results are shown in Table I (lines 9–12). In all these mutants, which are incapable of forming interchain disulfide bonds and are present as monomers (Fig. 3, lanes 1, 2, 4, and 5), the number of IRs at the cell surface was approximately half of that of IRs that can form disulfide bonds. Whereas these mutant IRs were clearly processed to α and β chains (Fig. 4, lane 4) and transported to the plasma membrane, there appeared to be a problem in the maturation process. The results are similar to those obtained with the mutant IR\text{C524S,C682S,C683S,C685S} that we had previously shown to be able to form noncovalent dimers (Table I, line 8; Fig. 1, lane 6; Fig. 4, lane 2).

Finally, we made IRs with Arg or Asp at both positions 524 and 682, 683, 685. In this case, there should be electrostatic repulsion at both inter-α chain contact surfaces. The results (Table I, lines 13 and 14) show that there was very little insulin binding activity at the cell surface, 6% of the IR\text{WT}, and that
and 13% of the amount of IR WT at the cell surface (Table I, proreceptor than did IRC524S,C682S,C683S,C685S (Figs. 2 and 4 lines 15 and 16), accumulated 1.5–2 times greater amounts of lines 7 visible (Fig. 1, panel B, IRC524W,C682S,C683S,C685S. Clearly any proreceptor made is rapidly destroyed. Although it was not possible to determine the insulin dissociation constant for these receptors due to the small amount of receptor, the immunofluorescence of cells expressing IRWT,C682R,C683R,C685R indicated that the cells do not have surface receptor so the lack of binding activity is not principally due to an increase in the value of the dissociation constant.

This situation is distinctly different from that of IRC524W and IRC524W,C682R,C683R,C685R, which although also present at only 12 and 13% of the amount of IR WT at the cell surface (Table I, lines 15 and 16), accumulated 1.5–2 times greater amounts of proreceptor than did IRC524S,C682S,C683S,C685S (Figs. 2 and 4A, lane 3). Under reducing conditions, the IRWT,C682S,C683S,C685S β subunit was identified with anti-V5 antibody (Fig. 4B, lane 3), suggesting that some proreceptors may have been processed. In these mutants, Trp at position 524 appears to prevent proper folding so that the issue of covalent or noncovalent interactions at the 682, 683, 685 positions is not relevant.

We conclude that proreceptor survival and processing requires covalent or noncovalent dimerization to be effective. If dimerization is prevented by electrostatic repulsion (Asp and Arg mutants), the proreceptor is rapidly destroyed; if the proreceptor is misfolded (Trp mutant), dimerization and processing are severely impaired even though dimerization might take place.

Post-translational Processing of Mutant IRs—To examine the processing stages of the three types of IRs, those that form covalent dimers, those that form noncovalent dimers, and those that are not processed, we carried out pulse-chase experiments with cells expressing IRWT, IRWT,C682R,C683R,C685R, and IRC524W,C682R,C683R,C685R. Fig. 5A shows the results obtained with IRWT and reproduces results already published (5). At the beginning of the cold-chase the primary product was proreceptor at position M1. After 45 min of chase, the proreceptor had shifted to position M2, corresponding to that of proreceptor with a difference in the number and/or arrangement of intramolecular disulfide bonds (19). At the same time the dimeric insulin receptors at positions D1 and D2 were visible. After 90 min, the M2 fraction was reduced with a concomitant increase in dimeric insulin receptors. After 3.5 h, only IR dimers were present.

Fig. 5B shows the results obtained with IRC524W,C682R,C683R,C685R, which can form noncovalent dimers. At the beginning of the cold chase, the protein was present as the proreceptor at positions M1 and M2. After 45 min, the amount of proreceptor M2 had increased, and curiously, there was also a band at position D1, corresponding to that of a covalent dimer even though all the canonical cysteine residues for inter-α chain disulfide bond formation are absent. After 90 min of cold-chase, the band at D1 disappeared concurrently with the appearance of the mature monomeric insulin receptor at position M3. After 3.5 h, the major product was mature monomeric insulin receptor. We conclude that during noncovalent dimerization of the proreceptor, some of the other cysteine residues in the α chain were utilized to form spurious inter-chain disulfide bonds. Because it is likely that this dimeric material was targeted for destruction, this process may explain the decreased number of IRs at the cell surface.

The situation with IRWT,C682R,C683R,C685R was simple and different from that in the preceding two cases. From the beginning of the cold-chase, there was material at position M of the proreceptor and at position D of a potential spurious dimer and no change in the pattern with time except for a nearly complete loss of all material at 3.5 h (Fig. 5C). The band at the position D was more diffuse and persisted for a longer period of time than was the case for the D1 band present with IRC524W,C682R,C683R,C685R. We suggest that the proreceptor at position D, either a spurious dimer or a monomer, is probably in a complex with endoplasmic reticulum chaperones making a diffuse band. The mature monomeric receptor (M3) was not detected in the cold-chase experiment, although a small amount of mature IRWT,C682R,C683R,C685R monomeric insulin receptor was observed by immunoblotting (Fig. 4, lane 3).

These results indicate that the stability of the IR depends on its ability to form covalent dimers. Although IRWT was stable after 7 h of chase, only 20% of IRC524W,C682S,C683S,C685S and less than 1% of IRC524W,C682R,C683R,C685R remained after 7 h.
Receptors that can form noncovalent dimers requires the intermediate of a covalent dimer of the proreceptor, thus explaining the presence of such a band in the case of IRC524S,C682S,C683S,C685S (Fig. 5B). If this were the case, one would expect that the spuriously dimeric receptor would be composed of H9251 and H9252 chains, whereas the monomeric proreceptor would not be processed. Accordingly, we examined the situation by two-dimensional SDS-PAGE under nonreducing and reducing conditions. The IRC524S,C682S,C683S,C685S labeled with 35S for 90 min was first separated under non-reducing conditions in a 3–10% gradient gel (Fig. 6A). The gel slice containing insulin receptors was then treated in Laemmli buffer containing 5% β-mercaptoethanol at 55 °C for 20 min and applied to the top of a second gel (B). The gels were analyzed with a Bio-Rad Molecular Imager FX system.

![Two-dimensional gel electrophoresis of IR C524S,C682S,C683,C685S labeled for 90 min.](image-a)

![Two-dimensional gel electrophoresis of IR C524S,C682S,C683,C685S labeled for 12 h.](image-b)

Monomeric Insulin Receptor Processing
transient; once the plasma membrane is saturated with IR, the proreceptor may no longer undergo further processing.

**Subcellular Distribution of Proreceptor and Receptor**—To determine the subcellular distribution of the three types of IRs, those that form covalent dimers, those that form noncovalent dimers, and those that are not processed, we separated the membrane compartments of COS7 cells by sucrose density sedimentation. The positions of the plasma membrane and the endoplasmic reticulum were identified by antibodies against the α chain of the Na,K-ATPase and against calnexin, respectively (17). As shown in Fig. 8, the plasma membrane was primarily present in the fractions containing 25–32% sucrose (Fig. 8A), whereas most of the endoplasmic reticulum was in fractions of 45–50% sucrose (Fig. 8D). In all three cases (Fig. 8, A–C), the proreceptors were exclusively detected in the fractions with high sucrose concentrations, indicating that they are localized in intracellular membranes, principally endoplasmic reticulum, which is consistent with previous observations (5). The β subunits from cells expressing IR WT (Fig. 8A) and IR C524S,C662S,C683S,C685S (Fig. 8B) were present both in intracellular membranes and in the plasma membrane, although the amount of β chain in the plasma membrane was greater for IR WT compared with IR C524S,C662S,C683S,C685S. In contrast, the β subunit from cells expressing IR C524W,C662S,C683S,C685S was detected only in the 41–52% sucrose fractions (Fig. 8C), supporting the result in Table I that a small number of IR C524W,C662S,C683S,C685S (12% of IR WT) are in the plasma membrane.

The cellular localization of IR WT and IR C524R,C662R,C683R,C685R was also examined by immunofluorescence staining. Fig. 9A shows that IR WT was abundant and present at the plasma membrane. On the other hand, the amount of IR C524R,C662R,C683R,C685R expressed was very small (Fig. 9B), as also demonstrated in Fig. 1, lane 8, and Fig. 3, lane 5, and it was present mainly in the intracellular compartments.

We conclude that the number of IR present in the plasma membrane is determined by the processing efficiency of the proreceptor, which depends on the dimerization ability of the proreceptor. To demonstrate the existence of proreceptor noncovalent dimers, we tried to cross-link the proreceptors of IR C524R,C662S,C683S,C685S in the endoplasmic reticulum in the same way that we cross-linked the mature IR C524R,C662S,C683S,C685S monomers in the plasma membrane (16). Although the proreceptor band did decrease in amount with increasing concentration of cross-linking agents, no clearcut proreceptor dimer band was evident; rather, a diffuse smearing of immunostaining material at higher molecular weights was observed (data not shown). It was not clear whether the shifts in position were caused by interaction with endoplasmic reticulum proteins associated with the proreceptor or by oligomerization of the insulin proreceptors themselves.

Finally, we compared the properties of the IR C524R,C662S,C683S,C685S and IR C524R,C662R mutants to those of the IR C524S,C662S,C683S,C685S mutant. The latter resembles IR WT in its ability to form covalent dimers and to appear at the plasma membrane (Table I, lane 4 and Fig. 1, lane 3). The question was whether a single Arg at position 682 was sufficient to prevent covalent dimerization through electrostatic interactions. The IR C524R,C662R mutant (Fig. 10, lane 3) was present at the plasma membrane in the same amount as the IR C524R mutant (Fig. 10, lane 2), and it formed covalent dimers, as did the IR C524R,C662S,C683S,C685S mutant (Fig. 10, lane 1). These results suggest that just 1 cysteine residue of the group at positions 682, 683, 685 is sufficient for covalent receptor dimerization even if there is some electrostatic interference. These results support the view that proreceptor dimerization is necessary for processing.

**DISCUSSION**

The biosynthesis of IR WT has been studied extensively. It has been proposed that proreceptors form disulfide-linked dimers and are then proteolytically cleaved into α and β subunits (3, 5). With an effective quality control system, most abnormal insulin receptors are retained in the endoplasmic reticulum and degraded.

The question of interest in this work is the mechanism of processing of IR proreceptors that are not capable of forming...
covalently linked dimers. We have shown previously that IRC524S,C682S,C683S,C685S is monomeric and is expressed at the cell surface at 50% of the amount of IRWT (16). Does processing of proreceptors require noncovalent dimerization?

To answer this question, we made mutant IRs in which the contact points between \( \alpha \) chains, residues 524 and 682, 683, 685, contain charged residues (Arg and Asp) that would be expected to prevent interaction. We first showed that substitution of Arg or Asp at 524 alone or at 682, 683, 685 alone had no effect on the appearance of the IR at the cell surface and that in all cases the IR was a dimer. Thus, processing and delivery to the plasma membrane are normal in these mutants. We conclude that Arg and Asp at 524 and at 682, 683, 685 do not affect folding of the molecule. However, when Arg or Asp is present at both 524 and 682, 683, 685, there is very little IR at the plasma membrane, and virtually no proreceptor is found in the cell. The simplest explanation is that the presence of the charged residues prevents formation of any interaction between the proreceptors, and thus, processing is prevented, and the proreceptor is destroyed. In contrast, if Ser is present at the contact positions or charged residues are present at only one of the contact sites with Ser at the other one, then noncovalent interactions can take place between proreceptors, and processing is allowed. One possible explanation for the reduced amount of processing in these cases is that the noncovalent interactions are weak, so only a fraction of the proreceptors is capable of dimerization. This conclusion is supported by the observation that the IRC524R,C682R mutant, in which there is reduced electrostatic repulsion at the 682, 683, 685 region, forms dimers and is expressed at a high level at the plasma membrane.

The situation is quite different for the IRC524W and IRC524W,S682,683,685S mutants, which we constructed with the idea that a bulky group might also interfere with dimerization. These mutants appear to be misfolded so that the proreceptors are not processed, they do not dimerize, and virtually no mature receptor appears at the cell surface. The amount of proreceptor of these mutants was at least equivalent to or slightly higher than that of the wild type receptor.

It was surprising that mutant monomeric insulin proreceptors also formed covalent dimers during post-translational processing (Fig. 5B). Because proreceptors dimerize before proreceptor cleavage (19), we carried out two-dimensional gel electrophoresis to determine whether the covalent dimer was required for proteolytic cleavage of proreceptor into \( \alpha \) and \( \beta \) subunits, an important step during insulin receptor maturation. Our results suggest that the covalent dimer observed during the pulse-chase experiment is not required for proteo-
lytic cleavage of proreceptor into α and β subunits (Fig. 6). It is more likely that proreceptors must form non-covalent dimers for processing and that the covalent dimers represent a small amount of spurious disulfide bond formation between non-canonical cysteine residues. Because insulin receptor monomers, IR524S,C682S,C683S,C685S, were cross-linked to dimers in the presence of insulin, indicating that the concentration of receptor monomers was high enough to form non-covalent dimers (16), it is not surprising that the proreceptors should also be able to dimerize.

We conclude that a correctly folded proreceptor has to dimerize to be processed into α and β chains. In the native state dimerization is stabilized by disulfide bonds between Cys-524, -682, -683, and -685. In the mutants lacking some of the Cys residues, the remaining ones are sufficient to allow covalent dimerization. The proreceptors of mutants, which have Ser at the positions of the Cys residues, form non-covalent dimers and are processed, but the extent of processing is decreased by mass action and possibly by spurious disulfide bonds between non-canonical cysteine residues. The proreceptors of mutants that cannot dimerize because of electrostatic repulsion are not processed and are rapidly destroyed.

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James Jianping Wu and Guido Guidotti

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